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(54) Title: METHOD FOR DETERMINING LACTIC ACID BACTERIAL SPECIES

(57) Abstract

The invention relates to a method for rapidly determining lactic acid bacterial species that are important as dairy starters or in probiotic use. The method is based on indicating the nucleic acid sequence characteristic of the bactrial species to be determined with a specific oligonucleotide or a pair of oligonucleotides. The species-specific oligonucleotides identifying the 16S-23S rRNA spacer region are described for the lactic acid bacterial species *Lactobacillus delbrueckii, Lb. (para* casei, Lb. acidophilus, Lb. helveticus and *Streptococcus thermophilus*.

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Method for determining lactic acid bacterial species

The invention relates to a method for determining, by means of gene technology, lactic acid bacterial species that are important as dairy starters or in probiotic use, and to species-specific oligonucleotides that are applicable in the method. The method enables fast detection of said lactic acid bacterial species.

Food industry is one of the most important branches of industry utilizing biotechnology. In addition to yeast, lactic acid bacteria are the most often used industrial microbes in food processing. Milk has a central position in Finnish agricultural production. Therefore the dairy industry that processes milk is also important in food industry. In Finland the most important soured milk products and cheese types are produced with starters containing lactic acid bacterial species that belong to the *Streptococcus*, *Lactococcus* or *Lactobacillus* group. So-called probiotic products where the lactic acid bacteria of the *Lactobacillus* group, in particular, have a central function have been introduced successfully into the market in recent years.

At present, lactic acid bacteria are identified and their amount in a sample is determined by microbiological, biochemical and physiological methods. Since the rate of cell division of lactic acid bacteria is rather low, it inevitably means that the determination of species by the aforementioned conventional methods is slow: the species determination of lactic acid bacteria from a sample typically takes a minimum of two days. In view of the requirements of monitoring the lactic acid fermentation processes, it is far too long. Since many industrial lactic acid

fermentation processes, for example the manufacture of yoghurt, are based on the simultaneous use of several species of lactic acid bacteria, the successful control of such processes requires as recent data as possible about the number of cells and the species distribution of the lactic acid bacteria. With the present methods these objects cannot be achieved in such a way that the same method would provide the required process control information both sufficiently rapidly and sufficiently specifically, but several methods must be used simultaneously so that they provide together the basic information needed.

Fast and relatively easy detection of microbes from a sample is possible on the basis of a sequence analysis of the nucleic acid material of microbial origin contained in the sample. The nucleic acid to be analyzed is usually DNA, since due to its biological instability prokaryotic RNA, in particular, is more difficult to analyze technically. The most commonly used technological alternatives include the use of specific oligonucleotides either as probes (so-called probe technology) or as primers in the DNA synthesis (so-called PCR technology). In both alternatives the method is based on indicating the identity or at least sufficient similarity of the genetic information (DNA nucleotide sequence) between the DNA contained in the sample under examination and the oligonucleotide (probe technology alternative) or the oligonucleotide pair (PCR technology alternative) used. As a biochemical manifestation of the genetic resemblance, specific hybridization occurs in both alternatives between the oligonucleotide and the complementary strand of the target DNA.

Especially advantageous in finding species-specific oligonucleotide probes or primer pairs have

proved to be the 5S, 16S and 23S rRNA genes of the ribosomal RNA operon (*rrn*) of the bacteria, and the intergenic i.e. the spacer regions thereof. Since due to the functional universality, the genes of the *rrn* operon contain conservative regions, *rrn* operon regions can be amplified by PCR with so-called universal primers also in cases where the actual sequence of the *rrn* operon or the parts thereof has not been identified before in the bacterial species under examination (Barry et al., *Biotechnology* 8 (1991) 233; Barry et al., *PCR Methods and Applications* 1 (1991) 51). Between the conservative operon regions, the degree of variation in the nucleotide sequence changes considerably from one bacterial species to another, and therefore the mutual phylogenetic positions of the bacterial species can be seen in the degree of homology of the nucleotide sequences in the variable regions of the *rrn* operons (Ludwig and Schleifer, *FEMS Microbiol. Rev.* 15 (1994) 155).

Species-specific oligonucleotide probes or primers identifying the *rrn* operon region, usually the 16S rRNA or 23S rRNA gene, have been recently developed for some industrially utilized lactic acid bacterial species, as well as for some harmful lactic acid bacterial species. With these oligonucleotides it has been possible to detect some *Lactobacillus* species used in the meat industry and in probiotic products (Hensiek et al., *System Appl. Microbiol.* 15 (1992) 123; Ehrmann et al., *FEMS Microbiol. Lett.* 117 (1994) 143) and some *Lactococcus*, *Streptococcus* and *Leuconostoc* species used in the dairy industry (Ehrmann et al., *System. Appl. Microbiol.* 15 (1992) 453). In Finland, the dairy industry produces milk or probiotic products where starter strains representing the species *Lactobacillus delbrueckii*, *Lactobacillus casei*, *Lactobacillus*

helveticus, Lactobacillus acidophilus and *Streptococcus thermophilus* have been used. Oligonucleotides that are suitable for the simultaneous and rapid determination of these lactic acid bacterial species and that identify species-specifically the spacer region of the 16S and 23S rRNA genes have not been described previously.

The object of the present invention is to provide a method which is based on the DNA technology and with which it is possible to determine rapidly and simultaneously the lactic acid bacterial species that are important as dairy starters and in probiotic use in Finland. The species determination of lactic acid bacterial species that are examined with the presently used microbiological pure culture methods and the subsequent biochemical species determination methods for the microbial material of the pure culture takes a minimum of 48 hours. The present invention provides a possibility of determining the species in less than one tenth of that time, which is highly advantageous especially for the monitoring and quality control of the production processes of milk and probiotic products.

The method according to the invention for determining, by means of gene technology, a lactic acid bacterial species that is important as a dairy starter or in probiotic use is characterized in that a nucleic acid sequence (target sequence) characteristic of said lactic acid bacterial species is detected from the 16S-23S ribosomal RNA (rRNA) spacer region of the bacterium. The method is based on detecting the sequence characteristic of the DNA of the bacterial species to be determined with a specific oligonucleotide or a pair of oligonucleotides.

The oligonucleotide according to the invention is characterized in that it hybridizes specifically with a nucleic acid sequence that is characteristic of a certain lactic acid bacterial species and that is from the 16S-23S rRNA spacer region of the bacterium. The oligonucleotides to be used are preferably derived from SEQ ID NO 1 to 9, and they are most preferably SEQ ID NO 10 to 19 or homologues thereof.

"Derived from" means either that the oligonucleotide has a counterpart in the sequence or in a complementary strand thereof, or that it is sufficiently similar with either one for specific hybridization with the target sequence in the species-specific determination. Therefore SEQ ID NO 11 to 19 are fragments of SEQ ID NO 2 to 9 or of the complementary strands thereof, whereas SEQ ID NO 10 contains the last nucleotide of the 16S rRNA gene, in addition to the fragment of SEQ ID NO 1.

A "homologue" of the aforementioned oligonucleotides refers to an oligonucleotide in which a few nucleotides may have been added, removed or replaced with another one, but which is sufficiently similar with the aforementioned oligonucleotides for hybridization specifically with the target sequence in the species-specific determination.

The preferred embodiments of the invention are disclosed in the dependent claims.

The present invention relates to a method which employs species-specific oligonucleotides for identifying, at the species-specific level, the DNA of one or several lactic acid bacterial species that are contained in the sample under examination and that are important as dairy starters or in probiotic use. The method is suitable for *Lactobacillus* and *Streptococcus thermophilus* bacteria that are important as dairy

starters and in probiotic use. One region that exists in all bacterial species and that has a degree of variation suitable for a potential species-specific nucleic acid sequence can be found from the spacer region of the 16S and 23S rRNA genes (Barry et al., *PCR Methods and Applications* 1 (1991) 51). Locating species-specific oligonucleotide sequences requires the determination of the nucleotide sequence of the target gene region from a suitably wide material of bacterial species and strains, if sequence data is not available otherwise, and the comparison of the nucleotide sequences, as regards the target gene regions, in order to detect the nucleotide sequences that are characteristic of the bacterial species.

The equivalent sequence to be detected from the sample with the species-specific nucleotide sequence may represent either a DNA sequence or an RNA sequence, depending on the method of preparation of the sample. Due to the biological instability and processing of RNA, it is easier to control DNA as the target nucleic acid with the method if the sensitivity requirements of the determination are otherwise fulfilled. In some applications which require great sensitivity of the method or biological activity of the sample, the selection of RNA as the target nucleic acid may be a better choice or even the only possibility.

The complementarity between the oligonucleotide and the target sequence is the basis for the specificity of the method, and therefore the conditions for hybridization and the subsequent processes should be standardized and optimally strict, taking into account the criteria of specificity and sensitivity of the method. The determination should include internal controls in the form of negative and positive reference samples. In the probe-technological method alternative,

one oligonucleotide is sufficient as a probe for indicating hybridization. In the PCR alternative, two oligonucleotides are needed as primers and they identify the complementary strands of the DNA segment to be amplified and extend the DNA synthesis they control reaching the complementary sequence of the other primer. In practice, the length of an oligonucleotide is usually about 15 to 25 nucleotides, and the length of the DNA segment to be amplified in PCR is optimally about 0.2 to 2 kb.

The requirement for species-specific identification provides that the oligonucleotide probe is species-specific and that at least one of the primers is species-specific whereas the other primer may be either species-specific or it may fulfil this specificity requirement less accurately. Example 3 shows the species-specific oligonucleotide sequences with which it is possible to indicate by PCR the corresponding lactic acid bacterial species from a sample in such a way that both primers are species-specific oligonucleotides. Pairs of oligonucleotide sequences are described for the following lactic acid bacterial species: *Streptococcus thermophilus*, *Lactobacillus delbrueckii*, *Lactobacillus (para)casei*, *Lactobacillus helveticus* and *Lactobacillus acidophilus*. According to the most recent taxonomy of lactic acid bacteria, the subspecies name *Lb. (para)casei ssp. casei* is replaced with the name [*Lb. paracasei ssp. paracasei*] and the subspecies name *Lb. casei ssp. rhamnosus* is replaced with the species name [*Lb. rhamnosus*]. In other words, the previous subspecies *ssp. casei* and *ssp. rhamnosus* are presently considered to belong to different species [*Lb. paracasei*] and [*Lb. rhamnosus*]. Species-specific oligonucleotides have also been described for these species. The names according

to the most recent taxonomy are given in brackets. The oligonucleotide sequences specific for each species are based on determining the nucleotide sequences of the 16S-23S rRNA spacer regions of the aforementioned bacterial species, and on analysing the obtained sequences.

Example 3 also shows a summary of the PCR results obtained with the species-specific oligonucleotides described in the invention for different types of materials of bacterial species. The results show that the oligonucleotide primer pairs described herein can be used to identify, species-specifically and simultaneously from the sample under examination, the lactic acid bacterial species that are important as dairy starters and in probiotic use in Finland, i.e. *Str. thermophilus, Lb. delbrueckii, Lb. casei, Lb. helveticus* and *Lb. acidophilus.*

The present invention provides the following advantages. The method enables a speed that is incomparably superior to the alternative methods for determining the aforementioned lactic acid bacterial species from a sample. The method enables the direct and simultaneous determination of several of the aforementioned lactic acid bacterial species from the same sample. The method also provides a large field of application both in research and in industrial processes and in the quality control thereof. Further, the method is safe to implement and sufficiently simple, and it can be realized with the usual up-to-date laboratory equipment, and it is economically competitive.

Example 1

Isolation of bacterial DNA

The bacterial strain was grown overnight in an appropriate culture medium. Cells were harvested by

centrifugation (14,000 g; 5 min) at 4°C and washed twice with 10 mM Tris-HCl buffer, pH 7.0. The washed bacterial cells were suspended in 10 mM Tris-HCl buffer (pH 7.0) containing 12% (w/v) PEG 6000 and 10 mg/ml of lysozyme enzyme (Sigma Chemical Co, St. Louis, USA), and the suspension was incubated for 30 min at 37°C. The cells were harvested by centrifugation (10,000 g; 15 min) at 4°C, lysis solution (10 mM EDTA-20 mM Tris-HCl buffer, pH 7.0, containing 3% (w/v) SDS) was added to the cell pellet, and the cell suspension was incubated for 30 min at 20°C. A sample of the disrupted cell solution was extracted twice with a phenol-chloroform-isoamylalcohol (25:24:1) mixture, whereafter the bacterial DNA was precipitated from the water phase with 200 mM NaCl and 50% (v/v) isopropanol. The precipitated bacterial DNA was harvested by centrifugation and washed with 70% ethanol, dried and dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

Example 2

Amplification and sequencing of the 16S-23S rRNA spacer region

The 16S-23S rRNA spacer region was amplified from the bacterial DNA by PCR (Saiki et al., *Science* 239 (1988) 487). The primers used for amplifying the DNA segment by PCR represented conservative regions at the end of the 16S rRNA gene and at the beginning of the 23S rRNA gene. The sequences of these oligonucleotide primers were: GTCGGAATCGCTAGTAATCG (primer 16-1A) and GGGTTCCCCCATTCGGA (primer 23-1B). The PCR reactions were carried out in a DNA Thermal Cycler 480 (Perkin Elmer, Norwalk, USA) using a DynaZyme DNA Polymerase kit (Finnzymes, Espoo, Finland).

A typical PCR reaction mixture contained sterile distilled water, reaction buffer (final concentrations 10 mM Tris-HCl, pH 8.8, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1 % Triton), 200 µM dNTP, 1 µM of each primer, 0.005 to 0.015 µg of bacterial DNA and 1.2 U (0.6 µl) of DynaZyme DNA polymerase solution/100 µl of reaction mixture. The volume of the reaction solution was typically 50 µl. A drop of mineral oil was added to each PCR reaction tube in order to prevent the evaporation of the reaction mixture.

The parameters of the PCR reaction cycle were: 30 sec at 95°C (denaturation), 30 sec at 55°C (annealing) and 30 sec 72°C (extention). The number of cycles was 30. Before the first cycle the reaction tubes were incubated for 2 min at 92°C. The PCR amplification was finished with 10 min extention at 72°C followed by cooling to 4°C. The PCR reaction products were analyzed by agarose gel electrophoresis. The excess primers and nucleotides were removed from the PCR reaction product by means of a QIAquick PCR purification kit (QIAGEN GmbH, Hilden, Germany).

Since in several cases the PCR amplification produced DNA fragments of different sizes (the genome of the bacteria has several *rrn* operons and the length of the 16S-23S rRNA intergenic region thereof varies), the DNA fragments of about 0.5 kb were isolated from the agarose gel and purified by means of a QIAquick gel extraction kit (QIAGEN GmbH, Hilden, Germany). The obtained DNA fragment was used as a template in the PCR amplification, which was carried out as described above. This PCR amplification only produced the expected DNA fragment of 0.5 kb and it was purified as described above.

The 16S-23S rRNA intergenic region was sequenced directly from the DNA fragments of about 0.5

kb obtained with the PCR amplification by cycle sequencing using a CircumVent Thermal Cycle Dideoxy DNA sequencing kit (New England Biolabs. Inc., Beverly, USA). The sequencing reactions employed 3 µl of the DNA solution (50 to 100 ng DNA) to be analyzed, and the reactions were carried out in the aforementioned PCR apparatus. The parameters of the sequencing reaction cycle were: 40 sec at 95°C, 30 sec at 55°C and 2 min at 72°C. The number of cycles was 15. The sequencing samples were analyzed by polyacrylamide gel electrophoresis. SEQ ID NO 1 to 9 were obtained. The nucleotide sequences are shown in accordance with the IUPAC standard.

<u>Example 3</u>

<u>PCR reactions with species-specific oligonucleotides, and analysis of PCR products</u>

The nucleotide sequence of the 16S-23S rRNA intergenic region of each examined bacterial species was analyzed to find the sequence regions specific for the species, and based on the sequence analysis, the oligonucleotides SEQ ID NO 10 to 21 (Table 1) suitable for use as species-specific primers were synthesized from both ends of the spacer region (the ends adjacent to the 16S rRNA and 23S rRNA genes).

Table 1

Species-specific oligonucleotide sequences

Oligo-nucleotide	SEQ ID NO:	Sequence (direction 5'->3')	Length (nt)	Direction (16S-23S)	Location (spacer)
LBA-AciI	10	TCTAAGGAAGCGAAGGAT	18	>>>>>	16S-end
-AciII	11	CTCTTCTCGGTCGCTCTA	18	<<<<<	23S-end
LBC-CasI	12	CAGACTGAAAGTCTGACGG	19	>>>>>	16S-end
-CasII	13	GTACTGACTTGCGTCAGCGG	20	<<<<<	23S-end
LBD-DelI	14	ACGGATGGATGGAGAGCAG	19	>>>>>	16S-end
-DelII	15	GCAAGTTTGTTCTTTCGAACTC	22	<<<<<	23S-end
LBH-HelI	16	GAAGTGATGGAGAGTAGAGATA	22	>>>>>	16S-end
-HelII	17	CTCTTCTCGGTCGCCTTG	18	<<<<<	23S-end
STH-ThI	18	ACGGAATGTACTTGAGTTTC	20	>>>>>	16S-end
-ThII	19	TTTGGCCTTTCGACCTAAC	19	<<<<<	23S-end
LBC-PcasII	20	GCGATGCGAATTTCTTTTTC	20	<<<<<	23S-end
LBC-RhaII	21	GCGATGCGAATTTCTATTATT	21	<<<<<	23S-end

The PCR reactions were carried out as described in Example 2, but instead of conservative primers, species-specific primer pairs were used (Table 1). The temperature of the annealing also varied depending on the primer pair as follows: 55°C for primer pairs AciI & AciII, CasI & PcasII, CasI & RhaII and ThI & ThII, 60°C for primer pair CasI & CasII, and 62°C for primer pairs DelI & DelII and HelI & HelII. The PCR reaction products were analyzed with agarose electrophoresis. Table 2 summarizes the reactions with each primer pair and each bacterial DNA tested.

According to the invention, it is also possible to distinguish the species [*Lb. paracasei*] and [*Lb. rhamnosus*] of the latest taxonomy for example with oligonucleotides PcasII (SEQ ID NO 20) and RhaII (SEQ ID NO 21), as shown in the last two columns of Table 2.

Table 2

PCR amplification of bacterial DNA with species-specific oligonucleotide primer pairs

Bacterial species & subspecies	Strain	PCR amplification with primer pair[a] LBC	LBD	LBA	LBH	STH	LBP	LBR
		SEQ ID NO 12&13	14&15	10&11	16&17	18&19	12&20	12&21
Lb. casei								
ssp. casei[b]	A-1[c]	+	-	-	-	-	+	-
ssp. rhamnosus[b]	G-1[c]	+	-	-	-	-	-	+
ssp. rhamnosus[b]	1/3[c]	+	-	-	-	-	-	+
Lb. paracasei								
ssp. casei	ATCC 27092[d]	+	-	-	-	-	+	-
Lb. delbrueckii								
ssp. lactis[b]	LKT[c]	-	+	-	-	-	-	-
	ATCC 15808[d]	-	+	-	-	-	-	-
ssp. bulgaricus[b]	LT4[c]	-	+	-	-	-	-	-
Lb. acidophilus	ATCC 4356[d]	-	-	+	-	-	-	-
Lb. helveticus	ATCC 15009[d]	-	-	-	+	-	-	-
helveticus[b]	H-1[c]	-	-	-	+	-	-	-
Str. thermophilus	ATCC 19987[d]	-	-	-	-	+	-	-
thermophilus[b]	ST-1[c]	-	-	-	-	+	-	-
Lb. salivarius								
ssp. salivarius	ATCC 11741[d]	-	-	-	-	-	-	-
Lactococcus								
lactis	JCM 7638[d]	-	-	-	-	-	-	-
lactis	P008-I(F7/2)[e]	-	-	-	-	-	-	-
Propionibacterium freudenreichii	ATCC 6207[d]	-	-	-	-	-	-	-
Escherichia coli	HB101[e]	-	-	-	-	-	-	-

[a] Primer pairs: LBC = CasI & CasII, LBD = DelI & DelII, LBA = AciI & AciII, LBH = HelI & HelII, STH = ThI & ThII, LBP = CasI & PcasII, LBR = CasI & RhaII.
[b] Species determination carried out with API 50CHL kit (BioMerieux S.A., France).
[c] Industrial lactic acid bacterial strain. Strain A-I proved to be identical to ATCC 27092.
[d] ATCC = American Type Culture Collection, JCM = Japan Collection of Micro-organisms.
[e] Laboratory strain.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Oulutech Oy
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(E) COUNTRY: Finland
(F) POSTAL CODE (ZIP): 90570

(ii) TITLE OF INVENTION: Method for determining lactic acid bacterial species

(iii) NUMBER OF SEQUENCES: 21

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 205 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Lactobacillus acidophilus
(B) STRAIN: ATCC 4356

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

CTAAGGAAGC GAAGGATATG GAGAGTAGAA ATACTAAGAG AAGTATCCAG AGCAAGCGGA 60

AGCACACTAA GAAACTTTGT TTAGTTTTGA GGGTAGTACC TCAAAAGAGT TAGTACATTG 120

AAAACTGAAT ATAATCCAAG CAAAAAACCG AGACAATCAA GAGAACAGAT TGTAGAGCGA 180

CCGAGAAGAG AATTCTTGGG TAAGG 205

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 222 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Lactobacillus casei ssp. rhamnosus [Lb. rhamnosus]
(B) STRAIN: 1/3

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

CTAAGGAAAC AGACTGAAAG TCTGACGGAA ACCTGCACAC ACGAAACTTT GTTTAGTTTT
60

GAGGGGATTA CCCTCAAGCA CCCTAGCGGG TGCGACTTTG TTCTTTGAAA ACTGGATATC
120

ATTGTTGTAA ATGTTTTAAA TTGCCGAGAA CACAGCGTAT TTGTATGAGT TTCTAATAAT
180

AGAAATTCGC ATCGCATAAC CGCTGACGCA AGTCAGTACA GG
222

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 221 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Lactobacillus casei ssp. casei [Lb. paracasei ssp. paracasei]
(B) STRAIN: A-1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CTAAGGAAAC AGACTGAAAG TCTGACGGAA ACCTGCACAC ACGAAACTTT GTTTAGTTTT
60

GAGGGGATCA CCCTCAAGCA CCCTAGCGGG TGCGACTTTG TTCTTTGAAA ACTGGATATC
120

ATTGTATTAA TTGTTTTAAA TTGCCGAGAA CACAGCGTAT TTGTATGAGT TTCTGAAAAA
180

GAAATTCGCA TCGCATAACC GCTGACGCAA GTCAGTACAG G
221

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 222 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Lactobacillus casei ssp. rhamnosus [Lb. rhamnosus]
(B) STRAIN: G-1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

CTAAGGAAAC AGACTGAAAG TCTGACGGAA ACCTGCACAC ACGAAACTTT GTTTAGTTTT
60

GAGGGGATTA CCCTCAAGCA CCCTAGCGGG TGCGACTTTG TTCTTTGAAA ACTGGATATC
120

ATTGTTGTAA ATGTTTTAAA TTGCCGAGAA CACAGCGTAT TTGTATGAGT TTCTAATAAT
180

AGAAATTCGC ATCGCATAAC CGCTGACGCA AGTCAGTACA GG
222

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 220 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Lactobacillus delbrueckii ssp. lactis
(B) STRAIN: ATCC 15808

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

CTAAGGAAAA CGGATGGATG GAGAGCAGAA ATGCTAARAG AAAGTCCATC AGTTACGGAA
60

GCACACTGCA AAAGAAACTT TGTTCAGTTT TGAGAGTATC AGCTCTCACT TGTACGTTGA
120

AAACTGAATA TCTTAATTCC AAGAAAAAAY CGAGAATCAT TGAGATCAAT GAAAACATTG
180

CAAAGCGACC GAGAGAGTTC GAAAGAACAA ACTTGCAAGG
220

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 222 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Lactobacillus delbrueckii spp. lactis
(B) STRAIN: LKT

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

CTAAGGAAAA CGGATGGATG GAGAGCAGAA ATGCTAARAG AAAGTTCCAT CCAGTTACGG
60

AAGCACACTG CAAAAGAAAC TTTGTTCAGY TTTGAGAGTA TCAGCTCTCA CTTGTACGTT
120

GAAAACTGAA TATCTTAATT CCAAGAAAAA AYCGAGAATC ATTGAGATCA ATGAAAACAT
180

TGCAAAGCGA CCGAGAGAGT TCGAAAGAAC AAACTTGCAA GG
222

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 222 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Lactobacillus delbrueckii spp. bulgaricus

(B) STRAIN: LT4

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

CTAAGGAAAA CGGATGGATG GAGAGCAGAA ATGCTAAGAR AAAGTCCATT CCAGTTACGG 60

AAGCACACTG CAAAAGAAAC TTTGTTCAGY TTTGAGAGTA TCAGCTCTCA CTTGTACGTT 120

GAAAACTGAA TATCTTAATT CCAAGAAAAA AYCGAGAATC ATTGAGATCA ATGAAAACAT 180

TGCAAAGCGA CCGAGAGAGT TCGAAAGAAC AAACTTGCAA GG 222

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 205 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Lactobacillus helveticus
(B) STRAIN: ATCC 15009

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

CTAAGGAAGC TGAAGTGATG GAGAGTAGAG ATACTAAGAG AAGTCACAAA AGCAAGCGGA 60

AGCACACTGA GAAACTTTGT TTAGTTTTGA GGGTAGTACC TCAAAGAGCT AGTACATTGA 120

AAACTGAATA TAATCCAAGC AAAAAACCGA GAAAATCAAA GAGAACAGAT TGCAAGGCKA 180

CCGAGAAGAG AATTCTTGAG TAAGG 205

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 274 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Streptococcus thermophilus
(B) STRAIN: ATCC 19987

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

CTAAGGAAAA ACGGAATGTA CTTGAGTTTC TTATTTAGTT TTGAGAGGTC TTGTGGGGCC
60

TTAGCTCAGC TGGGAGAGCG CCTGCTTTGC ACGCAGGAGG TCAGCGGTTC GATCCCGCTA
120

GGCTCCATTG AATCGAAAGA TTCAAGTATT GTCCATTGAA AATTGAATAT CTATATCAAA
180

TTCCATATGT AAGTAATTAC ATATAGATAG TAACAAGAAA ATAAACCGAA ACGCTGTGAA
240

TATTTAATGA GTTAGGTCGA AAGGCCAAAA ATAA
274

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Lactobacillus acidophilus

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

TCTAAGGAAG CGAAGGAT
18

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Lactobacillus acidophilus

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

CTCTTCTCGG TCGCTCTA
18

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Lactobacillus casei

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

CAGACTGAAA GTCTGACGG
19

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Lactobacillus casei

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

GTACTGACTT GCGTCAGCGG
20

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Lactobacillus delbrueckii

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

ACGGATGGAT GGAGAGCAG
19

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Lactobacillus delbrueckii

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

GCAAGTTTGT TCTTTCGAAC TC
22

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Lactobacillus helveticus

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

GAAGTGATGG AGAGTAGAGA TA
22

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Lactobacillus helveticus

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

CTCTTCTCGG TCGCCTTG
18

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Streptococcus thermophilus

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

ACGGAATGTA CTTGAGTTTC
20

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Streptococcus thermophilus

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

TTTGGCCTTT CGACCTAAC
19

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Lactobacillus casei ssp. casei [Lb. paracasei ssp. paracasei]

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

GCGATGCGAA TTTCTTTTTC
20

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Lactobacillus casei ssp. rhamnosus [Lb. rhamnosus]

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

GCGATGCGAA TTTCTATTAT T
21

Claims

1. A method for determining, by means of gene technology, a lactic acid bacterial species that is important as a dairy starter or in probiotic use, c h a r a c t e r i z e d in that a nucleic acid sequence characteristic of said lactic acid bacterial species is detected from the 16S-23S rRNA spacer region of the bacterium.

2. A method according to claim 1, c h a r a c t e r i z e d in that one or more lactic acid bacterial species are detected from the group consisting of *Lactobacillus delbrueckii*, *Lactobacillus (para)casei*, *Lactobacillus acidophilus*, *Lactobacillus helveticus* and *Streptococcus thermophilus*.

3. A method according to claim 1, c h a r a c t e r i z e d in that either one or both of *Lb. casei subsp. casei* and *Lb. casei subsp. rhamnosus* are detected specifically.

4. A method according to claim 3, c h a r a c t e r i z e d by using an oligonucleotide having SEQ ID NO 20 or 21.

5. A method according to claim 1, c h a r a c t e r i z e d in that the nucleic acid sequence is detected with a species-specific oligonucleotide or oligonucleotide pair that hybridizes specifically with the sequence.

6. A method according to claim 5, c h a r a c t e r i z e d in that the method employs PCR technology.

7. A method according to claim 6, c h a r a c t e r i z e d in that the primer pair is a pair of oligonucleotides that is derived from any one of SEQ ID NO 1 to 9.

8. A method according to claim 6 or 7, c h a r a c t e r i z e d in that the species-specific oligonucleotide is any one of SEQ ID NO 10 to 19.

9. Use of an oligonucleotide for determining lactic acid bacterial species that are important as dairy starters or in probiotic use, which oligonucleotide hybridizes specifically with a nucleic acid sequence that is characteristic of said lactic acid bacterial species and that is from the 16S-23S rRNA spacer region of the bacterium.

10. Use according to claim 9, c h a r a c t e r i z e d in that said oligonucleotide is derived from any one of SEQ ID NO 1 to 9.

11. Use according to claim 10, c h a r a c t e r i z e d in that said oligonucleotide is any one of SEQ ID NO 10 to 19.

12. Use according to claim 10, c h a r a c t e r i z e d in that said oligonucleotide is selected from SEQ ID NO 20 and 21.

13. An oligonucleotide, c h a r a c t e r i z e d in that it hybridizes specifically with a nucleic acid sequence that is characteristic of a lactic acid bacterial species that is important as a dairy starter or in probiotic use, and that is selected from the group consisting of *Lactobacillus delbrueckii*, *Lactobacillus acidophilus*, *Lactobacillus helveticus* and *Streptococcus thermophilus*, said sequence being from the 16S-23S rRNA spacer region of said bacterium.

14. An oligonucleotide according to claim 13, c h a r a c t e r i z e d in that it is derived from any one of SEQ ID NO 1 and 5 to 9.

15. An oligonucleotide according to claim 14, c h a r a c t e r i z e d in that it is any one of SEQ ID NO 10, 11 and 14 to 19.

16. An oligonucleotide, c h a r a c t e r i z e d in that it hybridizes specifically with a nucleic acid sequence which distinguishes the subspecies *Lb. casei subsp. casei* and *Lb. casei subsp. rhamnosus* from one another and which is from the 16S-23S rRNA spacer region of said bacterium.

17. An oligonucleotide according to claim 16, c h a r a c t e r i z e d in that it is selected from SEQ ID NO 20 and 21.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/FI 96/00471

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68 C07H21/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 60, no. 2, February 1994, pages 637-40, XP000609768 NAKAGAWA T ET AL: "Detection of alcohol-tolerant bacteria by PCR" see the whole document ---	1-17
Y	PCR METHODS AND APPLICATIONS, vol. 1, June 1991, pages 51-56, XP000609831 BARRY T ET AL: "The 16s/23s ribosomal spacer region as a target for DNA probes to identify eubacteria" cited in the application see the whole document ---	1-17
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

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Date of the actual completion of the international search	Date of mailing of the international search report
12 December 1996	07.01.97

Name and mailing address of the ISA	Authorized officer
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INTERNATIONAL SEARCH REPORT

Int' 'ional Application No
PCT/FI 96/00471

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP,A,0 452 596 (N.V. INNOGENETICS S.A.) 23 October 1991 see the whole document ---	1-17
Y	FEMS MICROBIOLOGY LETTERS, vol. 84, 1991, pages 307-312, XP000609758 KÖHLER G ET AL: "Differentiation of lactococci by rRNA gene restriction analysis" see the whole document ---	1-17
A	MILCHWISSENSCHAFT, vol. 48, no. 3, 1993, pages 123-125, XP000036493 TILSALA-TIMISJARVI A ET AL: "Enzymatic amplification of 16s rRNA gene sequences from dairy lactobacillus species" see the whole document -----	1,9,13

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/FI 96/00471

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		AU-B- 658143	06-04-95
		AU-A- 7755091	11-11-91
		CA-A- 2080812	19-10-91
		DE-D- 69113261	26-10-95
		DE-T- 69113261	13-06-96
		WO-A- 9116454	31-10-91
		EP-A- 0525095	03-02-93
		ES-T- 2080945	16-02-96
		JP-T- 5504889	29-07-93
		US-A- 5536638	16-07-96